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Technical note

Identifying lynx and other North American felids based on MtDNA analysis

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As part of a program to identify the distribution of Canada lynx (*Lynx canadensis*) across the U.S. using hair snags, we have developed a protocol to distinguish among all four felid species of northern North America (lynx, bobcat [*Lynx rufus*], cougar [*Felis concolor*], and domestic cat [*Felis catus*]) using mtDNA. Our tests were designed to be time and cost-efficient, and applicable to low-quantity or degraded DNA samples. Although it is possible to identify species using microsatellite DNA (e.g. Ernest et al. 2000), we favor mtDNA because of the greater copy number and because allele size constraints limit interspecific ranges of microsatellite DNA, potentially leading to allele frequency overlap (Nauta and Weissing 1996).

Because mtDNA is highly conserved among tissue types within an individual, and because tissue samples amplify more consistently than hair, we developed and validated our protocol using both tissue and hair samples. For hair samples, 5–10 follicles with shafts were typically used in extraction, although in some cases we successfully used single hairs with or without follicles (see below). Genomic DNA was extracted using standard protocols for tissues (Dneasy tissue kit; Qiagen Inc.), with overnight incubation in lysis buffer and Proteinase K on a rocker at 60 °C. Elution of DNA was in 50 μl of buffer.

To distinguish felid species from one another and from other species, we used the Polymerase Chain Reaction (PCR) to amplify two portions of the mitochondrial genome (Figure 1). One region includes the control region, amplified using conserved, universal primers L16007 and H16498 (Kocher et al. 1989; Shields and Kocher 1991). Twenty-μl PCR reactions contained 50–100 ng DNA, 1$\times$ reaction buffer (Perkin-Elmer), 2.5 mM $MgCl_2$, 200 μM each dNTP, 0.3 mg/ml BSA, 1 μM each primer, and 1 U *Taq* polymerase (Perkin-Elmer). After initial incubation at 94 °C for 5 min, the PCR profile was 35 cycles of 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 1.5 minutes. PCR products were run in a 2.0% agarose gel (Asubel et al. 1989).

The control region primers produced a PCR product of approximately 700 bp in all felid samples (Figure 1). This is the same region amplified by Foran et al. (1997a, b), except that primer L16007 results in a product 200 bp smaller, which we find amplifies with greater consistency. Felids have a 80–82 bp monomer within this region that may be heteroplasmic within individuals (Lopez et al. 1996), resulting in PCR products of approximately 700 bp, 780 bp, and 860 bp. However, while the PCR products from felid individuals may vary slightly (or have multiple products) the products are larger than that of other species (for example, the PCR product is 300–500 bp in mustelids, canids, ursids, and other species we analyzed).

As an independent mtDNA test, we amplified about 360 base pairs (bp) using 16S rRNA universal primers (Hoezel and Green 1992). Fifty-μl PCR reactions contained 50–100 ng DNA, 1$\times$ reaction buffer (Perkin-Elmer), 2.5 mM $MgCl_2$, 200 μM each dNTP, 1 μM each primer, and 1 U *Taq* polymerase (Perkin-Elmer). PCR profiles and gels were as for the control region, but with 50 °C annealing. We

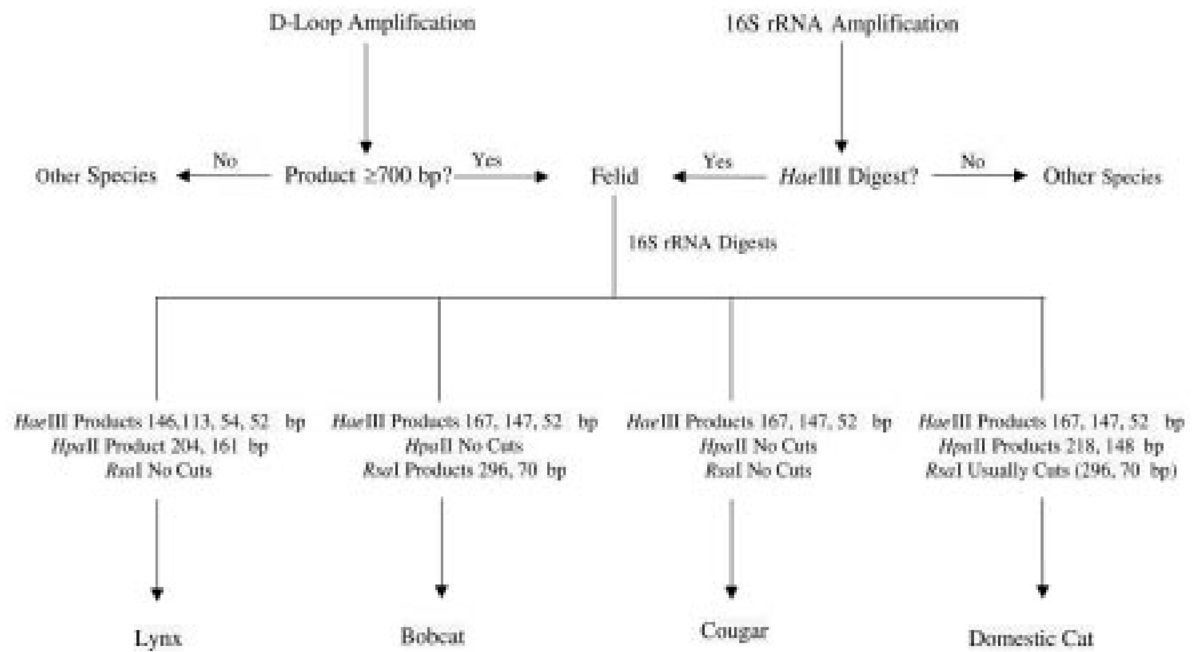

Figure 1. The approach used to genetically distinguish the felids from other species and from one another. Both tests of the D-loop region and 16S rRNA are used on all samples. Restriction digest analysis of 16S rRNA is conducted using all 3 enzymes to identify the four felid species.

developed restriction maps for the 16S rRNA region based on known sequences for lynx, bobcat, cougar and domestic cat (Johnson and O'Brien 1997). The restriction enzymes *Hae*III, *Hpa*II, and *Rsa*I digested the PCR products at 37 °C overnight, and fragments were visualized in a 2.5% agarose gel.

Like the control region, the 16S rRNA region with 3 restriction endonuclease digestions (*Hae*III, *Hpa*II, and *Rsa*I) separated felids from other species, but the 16S rRNA test also distinguished the four felids by species (Figure 2). Specifically, *Hae*III digested the PCR products from all four of the felids, but not any other species; there are three similar-sized products for the four felids, but lynx also produce a unique pattern of 113 bp. The restriction enzyme *Hpa*II does not digest bobcat or cougar PCR products, but causes unique product sizes for lynx and domestic cat. Finally, *Rsa*I digests PCR products from bobcat but not from lynx or cougar. This restriction site was polymorphic in domestic cats, with PCR products from 12 of 15 domestic cats tested cutting similar to bobcat.

We validated our protocol for identifying felids to species with a geographic range evaluation, a blind internal test, and a blind external test. The geographic range validation was intended to ensure that our protocol correctly identified cat species from many locations without being confounded by intra-specific polymorphisms. We successfully identified known samples of 34 lynx, 24 bobcat, 22 cougar, and 15 domestic cats from locations in Western Canada and 11 states across the U.S. The 'blind tests' assessed the reliability of the protocol both within our lab and in an independent lab (S. Fain, U.S. Fish and Wildlife Service Forensics Lab, Ashland, OR). Collectively, a total of 87 samples including 50 from felids (lynx, bobcat, cougar, domestic cat) and 37 from 18 other species (canids, ungulates, ursids, mustelids, rodents, lagomorphs, and humans) were correctly identified (felids to species and non-felids as 'other') 100% of the time.

Several species of *Felidae* contain mitochondrial-like sequences inserted into the nuclear genome (*Numt*; Lopez et al. 1996; Cracraft et al. 1998). However, the presence of *Numt* bands, which we have detected in approximately 20% of the 92 known lynx samples for which we have successfully amplified the control region segment, should not be a problem for species identification because the diagnostic mtDNA regions also amplify.

Following development and successful validation of the protocol, we have begun using hair snags (McDaniel et al. 2000) in a nationwide survey including 12 states across the northern U.S.

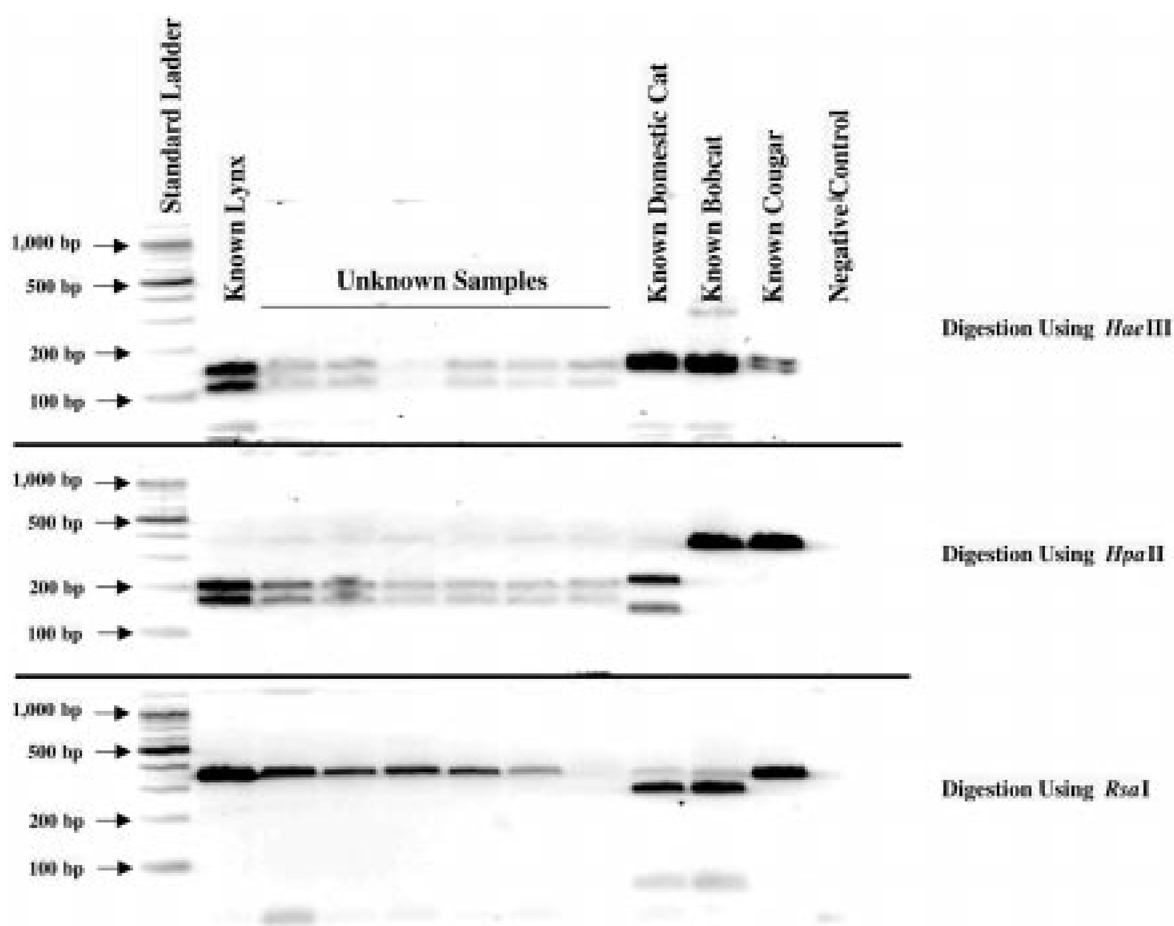

Figure 2. Agarose gel showing restriction digest analysis of the 16S rRNA region. The top panel shows PCR products digested using HaeIII, the middle shows PCR products digested using HpaII, and the bottom panel shows PCR products digested using RsaI. The first lane is a 1 kb size standard, followed by digested products from a known lynx. The next lanes are 4 hair samples and 2 blood samples collected in snow that were suspected to be from a lynx. The last three lanes are digested PCR products from a known domestic cat, bobcat, and cougar.

(McKelvey et al., in preparation). To date, 788 hair samples have been analyzed using the described protocol. We obtained DNA of high enough quality for amplification from 643 (82%) of these samples, and have detected all 4 felid species. Of the 643 samples that successfully amplified, 168 were single hairs; amplification was successful in 77% (46/60) of single hairs with follicles and 84% (91/108) without follicles. In short, our protocol conclusively identifies lynx and other sympatric felids (Figure 1) across geographic regions and across laboratories, even if samples are low quality or low quantity.

Diagnostic species identification using non-invasive hair sampling and mtDNA amplification leads to a remarkable set of tools for conservation. For the first time, geographic distribution across large spatial scales can be determined for many rare or elusive animals (such as Canada lynx, recently listed as Threatened in the contiguous U.S.). Furthermore, hair samples identified to species can in some cases be analyzed with the suite of emerging approaches for individual identification, phylogenetic analysis, and estimation of abundance and gene flow (Kohn and Wayne 1997; Schwartz et al. 1998; Taberlet and Luikart 1999; Mills et al. 2000).

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References

Asubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1989) *Current Protocols in Molecular Biology*. John Wiley & Sons, New York, New York, USA.

Cracraft J, Feinstein J, Vaughn J, Helm-Bychowski K (1998) Sorting out tigers (*Panthera tigris*): Mitochondrial sequences, nuclear inserts, systematics, and conservation genetics. *Animal Cons.*, **1**, 139–150.

Ernest HB, Penedo MC, May PB, Syvanen M, Boyce WM (2000) Molecular tracking of mountain lions in the Yosemite Valley region in California: Genetic analysis using microsatellites and fecal DNA. *Mol. Ecol.*, **9**, 433–441.

Foran DR, Crooks KR, Minta SC (1997a) Species identification from scat: An unambiguous genetic method. *Wildlife Soc. Bull.*, **25**(4), 835–839.

Foran DR, Minta SC, Heinemeyer KS (1997b) DNA-based analysis of hair to identify species and individuals for population research and monitoring. *Wildlife Soc. Bull.*, **25**(4), 840–847.

Hoelzel AR, Green A (1992) Analysis of population-level variation by sequencing PCR-amplified DNA. In: *Molecular Genetic Analysis of Populations: A Practical Approach* (ed. Hoelzel AR), pp. 159–187. IRL Press, Oxford.

Johnson WE, O'Brien SJ (1997) Phylogenetic reconstruction of the felidae using 16S rRNA and NADH-5 mitochondrial genes. *J. of Mol. Evol.*, **44**(Supplement 1), S98–S116.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX, Wilson AC (1989) Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proc. of the Nat. Acad. of Sci., USA*, **86**, 6196–6200.

Kohn MH, Wayne RK (1997) Facts from feces revisited. *Trends in Ecol. and Evol.*, **12**, 223–227.

Lopez JV, Cevario S, O'Brien SJ (1996) Complete nucleotide sequences of the domestic cat (*Felis catus*) mitochondrial genome and a transposed mtDNA tandem repeat (*Numt*) in the nuclear genome. *Genomics*, **33**, 229–246.

McDaniel GW, McKelvey KS, Squires JR, Ruggiero L (2000) Efficacy of lures and hair snares to detect lynx. *Wildlife Soc. Bull.*, **28**(1), 119–123.

Mills LS, Citta JJ, Lair K, Schwartz MK, Tallmon D (2000) Estimating animal abundance using non-invasive DNA sampling: Promise and pitfall. *Ecol. Appl.*, **10**, 283–294.

Nauta MJ, Weissing FJ (1996) Constraints of allele size at microsatellite loci: Implications for genetic differentiation. *Genetics*, **143**, 1021–1032.

Schwartz MK, Tallmon D, Luikart G (1998) Review of DNA-based census and effective population size estimators. *Animal Cons.*, **1**, 293–299.

Shields GF, Kocher TD (1991) Phylogenetic relationships of north American ursids based on analysis of mitochondrial DNA. *Evolution*, **45**(1), 218–221.

Taberlet P, Luikart G (1999) Non-invasive sampling and individual identification. *Biological Journal of the Linnean Society*, **68**, 41–55.